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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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A new infectious bursal disease virus variant

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A new infectious bursal disease virus variant

The present invention is concerned with a variant infectious bursal disease virus (IBDV) and a vaccine comprising such a variant IBDV.

Infectious bursal disease virus (IBDV) is a member of the Birnaviridae family. Viruses in this family have a very similar genomic organisation and a similar replication cycle. The genomes of these viruses consist of 2 segments (A and B) of double-stranded (ds) RNA. The larger segment A encodes a polyprotein, which is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4. VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of birnaviruses, and contains the immunogenic regions responsible for the induction of neutralising antibodies.

For IBDV, two serotypes exist, serotype 1 and 2. The two serotypes can be differentiated by virus neutralisation (VN) tests. Serotype 1 viruses have been shown to be pathogenic to chickens, while serotype 2 IBDV only causes sub-acute disease in turkeys. Infectious Bursal disease (IBD), also called Gumboro disease, is an acute, highly-contagious viral infection in chickens that has lymphoid tissue as its primary target with a selective tropism for cells of the bursa of Fabricius. The morbidity rate in susceptible flocks is high, with rapid weight loss and moderate to high mortality rates. Chicks that recover from the disease may have immune deficiencies because of the destruction of the bursa of Fabricius, which is essential to the defence mechanism of the chicken. The IBD-virus causes severe immunosuppression in chickens younger than 3 weeks of age and induces bursal lesions in chicks up to 3 months old.

For many years the disease could be prevented by inducing high levels of antibodies in breeder flocks by the application of an inactivated vaccine, to chickens that had been primed with attenuated live IBDV vaccine. This has kept economic losses caused by IBD to a minimum. Maternal antibodies in chickens derived from vaccinated breeders prevent early infection with IBDV and diminish problems associated with immunosuppression. In addition, attenuated live vaccines have also been used successfully in commercial chicken flocks after maternal antibodies had declined.

Historically, IBD viruses consisted of only one type that is known as "classic" IBD virus. However, in the mid-1980s acute disease in flocks vaccinated with vaccines based on classic IBDV was observed, in particular in the US. It was found that this disease was caused by IBD viruses that had a different immunogenic make-up. These new viruses probably emerged as a result of an antigenic shift. The emergence of these so-called "variant" IBDV strains required the design of new IBD vaccination programmes, because the classic IBDV vaccine strains

could not induce an adequate cross-protection. The most important variant subtypes of serotype 1 IBDVs identified in the past were the Delaware-E, GLS, RS/593 and DS326 variants.

Delaware variant-E was reported by Rosenberger et al. (Proc. 20th Natl. Meet. on Poultry Health and Condemnations; Ocean City, MD, USA, 94-101, 1985) and Snyder et al. (Avian Diseases 32, 535-539, 1985). GLS virus was isolated in the USA in 1987 and DS326 (GLS-like) was isolated in the USA in 1988 (Snyder et al., Arch. Virol. 127, 89-101, 1992 and van Loon et al. Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 179-187, 1994). Strain RS/593 (variant-E like) was also isolated in the USA, in 1993 (Snyder, et al. Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 65-70, 1994). In Europe, no emergence of IBDV variant strains has been reported yet.

A panel of virus neutralising monoclonal antibodies (Moab) is presently used in the art in an antigen-capture enzyme immuno assay (AC-ELISA) to identify the various IBDV types. The reactivity pattern of these Moabs with the existing IBDV strains is summarised in Table 1 below. Moreover, the molecular basis of the antigenic variation between IBDV strains has been elucidated by Vakharia et al. (Virus Research 31, 265-273, 1994). The region in the VP2 protein that displays most of the amino acid differences between IBDV strains and the putative amino acids involved in the formation of virus neutralising epitopes were identified.

Table 1

The different variant IBDV strains as determined by the Moab panel pattern

Strain↓/Moab→	8	B69	R63	10	BK9	67	57	44A1	179
Classic	+	+	+	+	-	-	-	+	+
Delaware variant (-E)	+	-	+	-	+	+	-	+	+
RS/593	+	-	-	-	-	+	-	-	+
GLS	+	-	-	+	-	-	+	+	+
DS326	+	-	-	+	-	-	+	+	-

VN Moabs R63 and B69 neutralize classic IBDV strains to high titres and Moab B69 specifically binds to classic strains. Moab BK9 uniquely binds to Delaware variant-E strains. A positive reaction by Moab 57 can be used to separate the GLS- and DS326 strains from classic- and Delaware variant strains. These and other Moabs are generally used in the field to distinguish between IBDV (variant) strains by determining the reaction pattern of the panel of available Moabs. The hybridomas secreting the Moabs are also available from the ATCC (Rockville, USA) under the following accession numbers: R63 (HB-9490), 8 (HB-10174), B29 (HB-9746), BK-9 (HB-10157), 67 (HB-11122), 57 (HB-10156), B69 (HB-9437) and 179 (HB-10158). Variant IBDV strains and hybridomas are also available from the Collection Nationale de Cultures de Microorganismes of the Institute Pasteur, Paris, France under the following accession no.'s: DS 326 (I-910), GLS (I-792 and I-793) and Moab 10 (I-2812).

In the present invention a new variant IBDV has been identified that was isolated from an outbreak of the disease that was not fully prevented by the administration of a classic vaccine. The new variant IBDV displaying an altered reaction pattern with the existing VN Moabs, disclosed for the first time herein, provides a basis for the preparation of new vaccines and diagnostic methods to prevent and monitor the disease caused by this new IBDV variant.

Therefore, the present invention provides a variant infectious bursal disease virus (IBDV), characterised in that the virus binds with monoclonal antibody 10 and 67, secreted by hybridoma cell lines I-2812 and HB-11122, deposited at the Collection Nationale de Cultures de Microorganismes of the Institute Pasteur, Paris, France and the ATCC, Rockville, USA, respectively.

Until now, in Europe, no IBDV strains different from the classic IBDV strain have been identified. This new IBDV representing a new variant strain was isolated from diseased animals in a flock that was vaccinated with a classic IBDV vaccine. The viruses belonging to this new strain are immunogenically different from classic- and known variant IBD viruses as demonstrated by their deviating Moab binding pattern.

Existing IBDV variant subtypes are well defined in the art, e.g. by means of their reaction pattern with monoclonal antibodies. The present variant IBDV lacks the 57 and BK9 epitopes that are characteristic for GLS- and variant-E strains, respectively. However, an IBDV according to the invention is a virus that comprises both the 10 and 67 epitopes. Moab 10 usually reacts with classical- and GLS-like strains, whereas Moab 67 only reacts with variant-E like strains.

The reaction of a Moab with the variant IBDV is determined by means of an AC-ELISA that is commonly used in the art for this purpose, such as described by Snyder et al. (1992, supra) or US patent no. 5,518,724. In the present invention the AC-ELISA as described by van

der Marel et al. (Dtsch. Tierartzl. Wschr. 97, 81-83, 1990) is used. This test basically comprises three steps. In a first step the antigenic mass of the IBDV antigen under investigation is examined by a Moab-ELISA using a Moab that reacts with IBDV, e.g. Moab B29. In a second step bursal homogenates of chickens infected with the IBDV are tested in an ELISA for their reactivity with a (relevant) panel of Moabs under standardized conditions. Thirdly, absorption ratios between the antigenic mass-Moab and the other Moabs are calculated.

It is generally accepted that biological variation exists in nature between organisms of the same species. For the purpose of this invention this means that different isolates may exist of the variant IBDV as defined above. These isolates comprise the 10 and 67 epitopes, but one or more nucleotide sequence differences may be observed in their genomes.

In particular, the present invention provides a variant IBDV as defined above that comprises codons for amino acids Ser (position 222), His (position 249), Ala (position 256) and Ser (position 278) in the VP2 coding region of segment A of the IBDV genome. The numbers indicating the amino acid- and nucleotide positions herein are based on the complete sequence of segment A of the IBDV genome as described by Mundt and Müller (J. Gen. Virol. 77, 437-442, 1995; NCBI accession no. X84034).

More preferably, the variant IBDV according to the invention comprises a VP2 coding region that encodes a VP2 protein having an amino acid sequence of the variable region variable region (aa 212-332) as shown in SEQ ID no. 1, more in particular, a VP2 coding region that encodes a VP2 protein having an amino acid sequence as shown in SEQ ID no. 1.

A most preferred virus according to the invention is IBDV isolate GB02, a sample of which is deposited at the Institute Pasteur, Paris, France under accession no. I-2811. Isolate GB02 PP represents plaque purified GB02 IBDV and is deposited at the Institute Pasteur, Paris, France under accession no. I-2925.

A variant IBDV according to the invention can be obtained from the Depository Institute or can be isolated from the field and identified by the above-mentioned Moabs as described in Example 1.

In a further aspect, the present invention provides an isolated polynucleotide comprising a nucleic acid sequence encoding a VP2 protein of a variant IBDV according to the present invention as defined above.

In particular, the polynucleotide comprises a coding sequence of the VP2 variable region having an amino acid sequence as shown in SEQ ID no. 1.

In a more preferred embodiment the polynucleotide encodes the VP2 protein having an amino acid sequence as shown in SEQ ID no. 1.

In addition to the coding sequence of the VP2 protein, the polynucleotide according to the invention may also comprise the coding sequences for the other structural proteins encoded by segment A of the IBDV genome, i.e. VP3 and VP4. cDNA clones containing the entire coding region of the segment A of the new variant IBDV can be prepared according to standard cloning procedures described in the prior art for this purpose (Vakharia et al., Avian Diseases 36, 736-742,1992; WO 91/1114, WO 95/26196 and US 5,595,912).

The present invention also provides an isolated polypeptide comprising the amino acid sequence of the VP2 protein of a variant IBDV according to the present invention, preferably as shown in SEQ ID no. 1. This polypeptide can be obtained by recombinant DNA expression using the polynucleotide defined above in a standard expression system. The VP2 protein of the new variant IBDV can be used in the form of a subunit protein or in the form of a virus-like-particle (VLP) when co-expressed with a VP3 and VP4 protein, preferably by a recombinant baculovirus, for the purpose of preparing an IBDV vaccine.

A wide variety of host cell and cloning vehicle combinations may be usefully employed in cloning the nucleic acid sequence according to the invention. A polynucleotide according to the invention may be cloned into an appropriate expression system, such as a bacterial expression system (e.g. Ecoli DH5α), a viral expression system (e.g. Baculovirus), a yeast system (e.g. S. Cerevisiae, Pichia) or eukaryotic cells (e.g. Cos, BHK, MDCK, MDBK, HeLa, PK15 cells). In all systems the polynucleotide is first cloned into an appropriate vector under control of a suitable constitutive or inducible promoter.

In another aspect the present invention therefore relates to a recombinant vector comprising a polynucleotide according to the invention. Suitable vectors are for example cosmids, bacterial or yeast plasmids, wide host range plasmids and vectors derived from combinations of plasmid and phage or virus DNA. Examples of suitable cloning vectors are plasmid vectors such as pBR322, the various pUC, pEMBL and Bluescript plasmids.

When used in the expression of the polypeptide, a recombinant vector according to the present invention, may further comprise an expression control sequence operably linked to the nucleic acid sequence coding for the protein.

All these techniques are well known in the art and extensively described in protocols provided by manufactures of molecular biological materials (such as Promega, Stratagene, Clonetech, and/or Invitrogen) and in literature or reference text books, for instance in Rodriguez, R.L. and D.T. Denhadt, edit., Vectors: A survey of molecular cloning vectors and their uses, Butterworths, 1988; Current protocols in Molecular Biology, eds.: F.M. Ausbel et al., Wiley N.Y., 1995; Molecular Cloning: a laboratory manual, 3rd ed.; eds: Sambrook et al.,

CSHL press, 2001 and DNA Cloning, Vol. 1-4, 2nd edition 1995, eds.: Glover and Hames, Oxford University Press).

As demonstrated in the Examples, the variant IBDV according to the invention displays an immunogenic make-up that is not observed before. The new variant IBDV may form the basis of a new type of IBDV vaccine that can effectively protect poultry against disease conditions resulting from the infection by the new variant IBDV. Therefore, another aspect of this invention is a vaccine for use in the protection of poultry against disease caused by IBDV infection, characterised in that the vaccine comprises a variant IBDV as defined above, together with a pharmaceutical acceptable carrier or diluent.

The variant IBDV can be incorporated into the vaccine as live attenuated or inactivated virus.

A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated IBDV vaccines. Briefly, a susceptible substrate is inoculated with a variant IBDV according to the invention and propagated until the virus replicated to a desired infectious titre after which IBDV containing material is harvested, optionally inactivated, and mixed with a pharmaceutical acceptable carrier or diluent.

Every substrate which is able to support the replication of IBDVs can be used to prepare a vaccine according to the present invention, including primary (avian) cell cultures, such as chicken embryo fibroblast cells (CEF) or chicken embryo liver cells (CEL), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation of the cells, the virus is propagated for 3-14 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

The variant IBDV can also be propagated in embryonated chicken eggs.

Attenuation of the variant IBDV can be obtained by standard serial passaging of the virus in cell cultures, for example in the primary cell cultures or established cell lines mentioned above (Bayyari et al., Avian Diseases 40, 516-532, 1996; Tsai et al., Avian diseases 36, 415-422, 1992).

Alternatively, the variant IBDV can be propagated in vivo in infected chickens followed by the isolation of the bursa of Fabricius from these infected animals, mixing it with diluent and homogenizing the mixture. IBDV propagated in this way commonly forms the basis of an inactivated vaccine.

The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilised form and additionally contains a

pharmaceutically acceptable carrier or diluent customary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below.

Although administration by injection, e.g. intramuscularly, subcutaneously or <u>in ovo</u> of the live vaccine according to the present invention is possible, the vaccine is preferably administered by an inexpensive mass application route commonly used for IBDV vaccination. For IBDV vaccination this route includes drinking water, spray and aerosol vaccination.

Alternatively, the present invention provides a vaccine comprising the variant IBDV in an inactivated (killed) form. An advantage of an inactivated IBDV vaccine is the high levels of protective antibodies of long duration that can be obtained.

The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means well known in the art.

A vaccine containing the inactivated variant IBDV can, for example, comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

The vaccine according to the invention comprises an effective dosage of the variant IBDV as the active component, i.e. an amount of immunising IBDV material that will induce immunity in the vaccinated birds against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

Typically, the live vaccine according to the invention can be administered in a dose of 10^0-10^9 TCID50 per animal, preferably in a dose ranging from 10^3-10^6 TCID50 per animal. Inactivated vaccines may contain the antigenic equivalent of 10^6-10^{10} TCID50 per animal.

Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

Although, the IBDV vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and partridges may be successfully vaccinated with the vaccine. Chickens include broilers, pullets, reproduction stock and laying stock.

The age of the animals receiving a live or inactivated vaccine according to the invention is the same as that of the animals receiving the conventional live- or inactivated IBDV vaccines. For example, broilers (free of maternally derived antibodies-MDA) may be vaccinated at one-day-old or in ovo, whereas broilers with high levels of MDA are preferably vaccinated at 2-3 weeks of age. Laying stock or reproduction stock with low levels of MDA may be vaccinated at 1-10 days of age followed by booster vaccinations with inactivated vaccine on 6-12 and 16-20 weeks of age.

The invention also includes combination vaccines comprising, in addition to the variant IBDV described above, one or more vaccine components of other pathogens infectious to poultry.

Preferably, the combination vaccine additionally comprises one or more vaccine strains of Mareks Disease virus (MDV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV) or reovirus. Alternatively, the combination vaccine additionally comprises an IBDV vaccine strain of different immunogenic make-up, such as a classic-, variant E or GLS vaccine strain.

EXAMPLES

Example 1 Isolation and characterisation of the variant IBDV

Materials and Methods

Isolation of the new IBDV from Bursa material of infected chickens

The new virus was isolated from bursas of diseased animals according to the following procedure:

A chicken flock vaccinated with classic vaccine showed wet litter, general immunosuppression, low weight gain but no high mortality (1-2%). Chickens from this flock were offered for post-mortem examination. Some muscle bleedings were seen and some small bursae. Bursae were isolated, PBS and antibiotics added. Next, the bursae were homogenized with glass pearls and sterile PBS. The homogenates were tested using the Moab panel test. IBDV material that showed a deviating panel pattern was passaged further in animals. 0.1 ml of this homogenate was applied via the in eye-drop route to 14 days old SPF white leghorns. Three-4 days after inoculation the presence of IBDV in the bursa of Fabricius was investigated using the Moab panel test.

Primary chicken embryo fibroblasts (CEF) cells were prepared at a final concentration of 2×10^6/ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 15 ml of this cell suspension 0.1 ml IBDV isolate GB02 (at passage level 1) was added. After incubation for 3-6 days in a high humidity incubator at 37°C, the supernatant contained the virus strain. After 2 passages on CEF the virus was further purified by three plaque purification rounds on CEF. Next the virus was cultured in the same way as described above on CEF for 2 rounds. A sample of the plaque purified GB02 isolate (GB02 PP) was deposited at the Institute Pasteur, Paris, France under accession no. I-2925.

IBDV GB02 after 3 plaque purification rounds on CEF was cultured for 2 passages on VERO cells. After the second incubation on VERO cells for 10 days at 37°C the infected cells suspension was filtered through a sterile cheese cloth.

Antigen-capture enzyme immuno assay (AC-ELISA)

IBDV was characterised by means of an ELISA using different monoclonal antibodies according to van der Marel et al. (Dtsch. Tierartzl. Wschr. 97, 81-83, 1990):

1. Moab B29 was coated on 96 well microtiterplates. After coating, the plates were washed and the wells of the plates were filled with two- or threefold dilution series of bursal homogenate samples and a standard antigen of known antigenic mass. After incubation and washing of the plates, the plates were incubated with a hyperimmune rabbit anti-IBDV serum. Next the plates were washed again and incubated with conjugate (goat-anti-rabbit immunoglobulin coupled to horse-radish-peroxidase). Finally, the plates were incubated with substrate solution. The enzymatic reaction was stopped with 4N H_2SO_4.The absorption in the wells was read at 450nm with an ELISA reader. The antigenic mass content of the bursal samples relative to the standard antigen was calculated by regression analysis. The antigenic content of the bursal samples is expressed as B29 EU/ml.

2. The subsequent procedure was similar to the one described for the B29 ELISA with the exception of: 1. The plates were coated with different Moabs, 2. The bursa homogenate samples were diluted to a concentration of 5000 B29 EU/ml. Then a single dilution was dispensed in the wells coated with the different Moabs. The subsequent steps were the same as for the B29 ELISA.

3. The absorption ratios were calculated according to the following formula

$$\text{Ratio} = \frac{\text{E450 Moab “X”} - \text{E450 background of Moab “X”}}{\text{E450 Moab B29} - \text{E450 background of Moab B29}}$$

The ratios are classified as follows:

Ratio	reactivity	explanation
0-0.3	-	Moab does not bind to virus strain
0.3-0.7	0	reduced Moab binding to virus strain
>0.7	+	complete Moab binding to virus strain

Cloning and sequencing of segment A of the variant IBDV

Material and Methods

Purification of viral RNA.

Obtained bursae of chicken were homogenized. The homogenate was centrifuged at 13000 x g for 5 min to eliminate cellular debris. One volume of chloroform were added to the obtained supernatant and vortexed. After centrifugation at 13000 x g for 5min to the resulting supernatant proteinase K and sodium dodesylsulphate was added to a final concentration of 1mg/ml and 0.5%, respectively, and incubated at 56°C for 1 hour. Nucleic acids were obtained after extraction of the digested proteins using one volume phenol/chloroform. After one additional phenol extraction using one volume chloroform nucleic acids were precipitated by adding of 1/10 volume of 3M sodium acid (pH5.2) and 2.5 volume absolute ethanol.

Construction of a cDNA clone comprising segment.

For cloning of the cDNA of segment A the purified RNA was reverse transcribed into cDNA, and amplified by polymerase chain reaction (PCR) following standard procedures using the methods as described by Mundt and Vakharia (PNAS 93, 11131-11136, 1996).

Appropriate primer pairs (BelgFP/BelgRP nucleotides 608-629 and 1201-1222) were added to dissolved nucleic acids. The primer-nucleic acid mixture was added to the reaction mixture [reaction buffer (Invitrogen), DTT (Invitrogen), dNTP mixture (Promega), Superscript II (Invitrogen)],

The amplification of the appropriate cDNA fragments were performed using the primer pair BelgFP/BelgRP resulting in the RT-PCR fragment BelgApart. The PCR was performend by using Deep Vent polymerase (New England Biolabs) and 2µl of the RT-reaction.

Amplification product was cloned blunt ended and plasmids containing appropriate PCR fragments were sequenced. The cloning procedure to obtain a plasmid containing the segment A under control of the T7- RNA polymerase promotor corresponded to the procedure described by Mundt and Vakharia (1996, supra).

Three plasmids resulting from cloning of each PCR fragment (pBelgApart) were analysed by sequencing of the DNA in both directions. Cloned PCR fragments were sequenced and obtained sequences were analysed using the Wisconsin package, Version 8 (Genetics Computer Group, Madison, Wis., USA).

The amino nucleotide sequence as well as amino acid sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw).

Results

A new immunogenic variant IBDV was isolated from the bursa material. This virus was designated isolate GB02. The panel pattern for this new variant IBDV isolate is shown in Table 2A. Results of controls samples are given in Table 2B.

Table 2A: Panel pattern of new variant IBDV with different Moabs.

Sample↓/Moab→	8	R63	B69	10	BK9	67	57	44A1
GB02	+	+	+	+	-	+	-	ND

Table 2B: Panel pattern of different IBDVs with different Moabs.

Controls↓/Moab→	8	R63	B69	10	BK9	67	57	44A1
Classic (F52/70)	+	+	+	+	-	-	-	+
Variant-E	+	+	-	-	+	+	-	+
GLS	+	-	-	+	-	-	+	+

+ epitope present on virus, - epitope not present on virus. ND = not done.

From Tables 2A and 2B it can be seen that the newly isolated IBDV reacted in a different way with the different monoclonal antibodies than the control samples. The new virus reacts with both Moab10 and Moab 67. Moab 10 normally reacts with classic and GLS-like strains but never in combination with variant-E-like viruses. Moab 67 reacts only with variant-E-like viruses and never with classic and GLS-like viruses. The combination of Moab 10 and 67 present on the same virus at the same time is a unique combination, indicating that the isolated virus is a new IBDV variant.

A sample of the new variant IBDV of the (2nd) CEF passage was deposited at the Collection Nationale de Cultures de Microorganismes of the Institute Pasteur, Paris, France under accession no. I-2811.

Furthermore, a cDNA clone comprising the VP2 gene of the new variant IBDV was generated and sequenced. The nucleotide sequence and the deduced amino acid (aa) sequence is shown in SEQ ID No. 1-2.

It is known that the epitopes that specifically react with the various Moabs are located on the VP2 protein, in particular, in the so-called variable regions that generally spans amino acids 212 to 332 (see Vakharia et al., 1994, supra).

The obtained partial nucleotide sequence of segment A was compared with sequences of different strains of IBDV (Table 3).

<u>Table 3</u>

```
                 174       184       194       204       214       224
GB02       VLSLPTSYDLGYVRLGDPIPAIGLDPKMVATCDSSDRPRVYTITAADDYQFSSQYQSGG
52-70      --------------------------------------------------------P--
002-73     --------------------------------------------------------P--
UK661      --------------------------------------------------------A--
Var-A      --------------------------------------------------------Q--
Var-E      -----------------------------------------------N--------T--
GLS        --------------------------------------------------------T--

                 234       244       254       264       274       284
GB02      VTITLFSANIDAITSLSIGGELVFHTSVQGLALNATIYLIGFDGTTVITRAVASDNGLTT
52-70     ------------------------Q------V-G-----------A-------A-----A
002-73    --------------N-V-------Q------V-------V-------T-----AG----A
UK661     ------------------------Q------I-G-----------A-------A-----A
Var-A     -----------------V------K----S-V-G-----------A-------AN----A
Var-E     -----------------V------K----S-V-G-----------A-------AN----A
GLS       -----------------V------K---HS-V-G----------SA-------AN-----

                 294       304       314       324       334       344
GB02      GIDNLMPFNLVIPTNEITQPITSIKLEIVTSKSGGQAGDQMSWSASGSLAVTIHGGNYPG
52-70     -T----------------------------------------------------------
002-73    -T------------S-----V----------------------L---N------------
UK661     -T-------I----S---------------------------------------------
Var-A     ---------------------------------D--------------------------
Var-E     ---------------------------------D----E---------------------
GLS       -T----------------------------------------------------------

                 354
GB02      ALRPVTLVAYER
52-70     ------------
002-73    ------------
UK661     ------------
Var-A     ------------
Var-E     ------------
GLS       ------------
```

Alignement of amino acid sequences of different serotype I strains of IBDV. The partial amino acid sequence of the belgium GB02 isolate encoded by the polyprotein gene of segment A was aligned with sequences of the Variant E strain (Var-E: Genbank accession number X54858), Variant A strain (Var-A: M64285), the australian strain 002-73 (Nucleic Acids Research, 1986, 12, pp.5001-5012), the very virulent strain UK661 (AJ318896), the classical strain Faragher 52-70 (D00869), and the variant strain GLS (M97346). The numbering of the amino acid sequence in in accordance to Mundt and Müller (supra, 1995).

The compared region showed a variety of amino acid exchanges to all sequences used for the sequence comparison. This is further evidence that the newly isolated virus represents a new type of IBDV not described so far. The highest number of exchanges was detected in the VP2 variable region (aa 212-332). The comparison of the percentage homology of the nucleotide- and amino acid sequences (92.3% to 95.9%) showed that the sequences of the new virus were as much different to the subtypes of IBDV as the subtypes were different to each other.

<u>Example 2</u> Vaccination of chickens with a vaccine based on the variant IBDV

Experimental design

Two groups of 20 three-week-old SPF chickens were vaccinated with inactivated variant IBDV vaccine or inactivated classical IBDV vaccine. Another group of 20 three-week-old SPF chickens was not vaccinated to serve as control. At 6 weeks post vaccination, all vaccinated and control chickens were subjected to a challenge with the IBDV variant field isolate. At four days post challenge the bursae were removed and examined for the presence/absence of IBDV variant challenge virus in an antigen capture ELISA.

Before the start of the experiment blood samples were collected from 20 chickens. At 2, 4 and 6 weeks post vaccination blood samples were collected from all chickens individually. Sera were examined for the absence/presence of IBDV antibodies in a virus neutralisation (VN) test.

Materials and Methods

<u>IBDV variant vaccine</u>

Variant IBDV (isolate GB02) was produced on VERO cells and subsequently inactivated with formaldehyde. The inactivated IBDV GB02 antigen was emulsified in a W/O emulsion so that each dose contained 10 EU (R63 ELISA based) of IBDV GB02. Chickens were vaccinated each with 0.5 ml of the IBDV GB02 vaccine via intramuscular route in the leg muscle.

IBDV classical vaccine

Classical IBDV (strain D78) was produced on VERO cells and subsequently inactivated with formaldehyde. The inactivated IBDV D78 antigen was emulsified in a W/O emulsion so that each dose contained 10 EU (R63 ELISA based) of IBDV D78. Chickens were vaccinated each with 0.5 ml of the IBDV D78 vaccine via intramuscular route in the leg muscle.

Challenge material

IBDV GB02 was plaque purified on CEF and subsequently passaged in SPF chickens. Chickens were challenged each with a calculated infectivity titre of $10^{5.1}$ $TCID_{50}$ of GB02 challenge virus via the ocular route.

Serology

Sera were examined for the presence/absence of IBDV antibodies in a virus neutralisation test according to the procedure outlined below.

Serial two-fold dilutions of the sera were mixed with an equal volume of virus containing approximately 750 $TCID_{50}$ of either IBDV D78 or IBDV GB02. Following incubation of 90 minutes at +37°C chicken embryo fibroblasts (CEF) were added. The antibody titres were established after 5 days of incubation at +37°C by microscopical examination of the CEF monolayers for the presence/absence of CPE characteristic of IBDV. Antibody titres were the reciprocal of the highest dilution in which the IBDV was neutralised completely. Serum samples with titres of <4 (log_2) were considered negative for IBDV antibodies.

Antigen capture ELISA

Two dilutions (1:2 and 1:4) of the bursa homogenates were added to IBDV monoclonal antibody MCA 67 absorbed onto a microtitre plate. Following incubation of 1.5 hours at +37°C, the presence of IBDV GB02 challenge virus bound to MCA67 was detected by adding IBDV monoclonal antibody MCA8 coupled to horse radish peroxidase. Samples with absorbance values higher than 2 times the mean back ground value were considered positive for IBDV GB02 challenge virus.

Results and discussion

Serology

No antibodies to IBDV D78 and IBDV GB02 could be detected in the sera obtained before the start of the experiment.

The mean IBDV antibody titres determined in the sera collected at 2, 4 and 6 weeks post vaccination outlined in the table below:

Table 4:

Inoculum	Mean IBDV VN antibody titre (standard deviation) at ... weeks pos vaccination					
	2 wks		4 wks		6 wks	
	D78	GB02	D78	GB02	D78	GB02
IBDV D78 (10 EU/dose)	3.8 (1.3)	<4.0 (0.0)	11.6 (3.2)	6.0 (2.4)	12.8 (2.9)	6.0 (2.3)
IBDV GB02 (10 EU/dose)	5.8 (2.2)	6.3 (1.3)	9.4 (2.4)	12.0 (2.3)	9.7 (1.6)	14.0 (2.5)
Controls (no inoculation)	<4.0 (0.0)	<4.0 (0.0)	<4.0 (0.0)	<4.0 (0.0)	<4.0 (0.0)	<4.0 (0.0)

These results show that both the inactivated IBDV D78 vaccine and the inactivated IBDV GB02 vaccine provoke an immune response following vaccination of three-week-old SPF chickens. Following vaccination with the classical inactivated IBDV D78 vaccine, the cross neutralisation test revealed a relatively large difference in mean antibody titre against the homologous IBDV D78 antigen and the heterologous GB02 antigen. The latter indicates that the new variant IBDV is not antigenically related to the classical IBDV.

Detection of IBDV challenge virus in the bursa by an antigen capture ELISA

Results obtained with the detection of challenge virus in the bursae removed at 4 days post challenge are outlined in the table below.

Table 5:

Inoculum	Percentage of bursae in which IBDV challenge virus was detected
IBDV D78 (10 EU/dose)	40%
IBDV GB02 (10 EU/dose)	0%
Controls (no inoculation)	90%

The results show that after challenge with the IBDV GB02 field isolate no challenge virus could be detected in any of the bursae derived from the chickens vaccinated with the inactivated IBDV GB02 vaccine. On the other hand, IBDV GB02 challenge virus could be detected in 40% of the bursae derived from chickens vaccinated with the inactivated IBDV D78 vaccine.

As the bursa is considered the predilection site of virus replication for IBDV, the results obtained in this experiment indicate that the inactivated IBDV GB02 vaccine provides chickens with a solid protection against infection with the IBDV GB02 field isolate.

Thus, the results indicate that a vaccine based on a classical IBDV does not provide chickens with a solid protection against infection with the new variant IBDV.

CLAIMS

1 A variant infectious bursal disease virus (IBDV), characterised in that the virus binds with monoclonal antibody 10 and 67, secreted by hybridoma cell lines I-2812 and HB-11122, deposited at the Collection Nationale de Cultures de Microorganismes of the Institute Pasteur, Paris, France and the ATCC, Rockville, USA, respectively.

2 A variant IBDV according to clam 1, characterised in that it comprises codons for amino acids Ser (position 222), His (position 249), Ala (position 256) and Ser (position 278) in the VP2 coding region of segment A of the IBDV genome.

3 A variant IBDV according to claim 2, characterised in that it comprises a VP2 coding region that encodes a VP2 protein having an amino acid sequence of the variable region as shown in SEQ ID no. 1.

4 A variant IBDV according to claim 3, characterised in that it is virus isolate GB02, a sample of which is deposited at the Institute Pasteur, Paris, France under accession no. I-2811.

5 A vaccine for use in the protection of poultry against disease caused by IBDV infection, characterised in that the vaccine comprises a variant IBDV according to claims 1-4, together with a pharmaceutical acceptable carrier or diluent.

6 A vaccine according to claim 5, characterised in the variant IBDV is in an inactivated form.

7 A vaccine according to claims 5 or 6, characterised in that the vaccine further comprises one or more vaccine components of other pathogens infectious to poultry.

8 A vaccine according to claims 5-7, characterised in that the vaccine comprises an adjuvant.

9 A method for the preparation of the variant IBDV according to claims 1-4, characterised in that the variant IBDV is propagated in a cell culture and subsequently harvested from the cell culture.

10 A method for the preparation of a vaccine according to claims 5-8, characterised in that a variant IBDV according to claims 1-4 is mixed with a pharmaceutical acceptable carrier or a diluent.

11 A method for the protection of poultry against disease caused by IBDV infection, characterised in that the vaccine according to claims 5-8 is administered to the animals.

ABSTRACT

The present invention provides a variant IBDV that is distinguished from the known IBDV (sub-) types, such as classic-, GLS- and variant-E IBDV.

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